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ESTERIFICATION OF ARYLHYDROXYLAMINES: EVIDENCE FOR A SPECIFIC GENE PRODUCT IN MUTAGENESIS

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Characterization of a Salmonella typhmurium mutant strain (TA98/1,8-DNP$_6$) resistant to the mutagenicity of nitrated polycyclic aromatic hydrocarbons (nitroarenes) revealed that it was also non-responsive to the mutagenic action of nitroso- and N-hydroxylaminoarenes. The mutant strain was fully sensitive to the mutagenic action of the corresponding hydroxamic acid ester. These results suggest that TA98/1,8-DNP$_6$ is deficient in a specific esterifying enzyme and that esterification of the penultimate mutagenic metabolites of nitro- and aminoarenes (e.g., arylhydroxylamines) to form potent electrophiles is controlled by a specific gene.

Introduction

The recognition that many emissions from incomplete combustion processes, notably diesel engines, contain nitroarenes which are potent microbial mutagens, has resulted in a number of studies seeking to determine the chemical basis for this unusual biological activity (1). An undertstanding of this phenomenon seems especially timely in view of the projection that within 10 years in excess of 25% of passenger automobiles will be diesel-powered (2,3). Urgency was added when it was found that some of these nitroarenes are also carcinogenic for animals (1,4).

In bacteria the biological activity of nitroarenes seems to depend upon their conversion to arylhydroxylamines, which are either direct-acting or become so following esterification to electrophiles. A number of findings support this reductive activation: (a) The mutagenic activity of nitroarenes is greatly decreased in nitroreductase-deficient microorganisms. (b) This nitroreductase deficiency can be over-

Abbreviations

AAF, acetylaminofluorene; N-AcAAF, N-acetoxy-2-AAF; BMBA, 7-bromomethyl-12-methylbenz(a)anthracene; DMBA, 7,12-dimethylbenz(a)anthracene; S9, post-mitochondrial fraction.

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come when the nitroarenes are replaced by the corresponding arylhydroxylamines. (c) Incubation of nitroreductase-proficient bacteria in the presence of radioactive nitroarenes results in the accumulation of the corresponding amino- and N-acetylaminoarenes. This accumulation is greatly reduced in nitroreductase-deficient bacteria. (d) Adduct formation between radioactive nitroarenes (e.g., 1-nitropyrene) and the bacterial DNA is greatly decreased in nitroreductase-deficient bacteria (1,5,6).

Mammalian cells are also susceptible to the mutagenic and genotoxic actions of nitroarenes. However, this may occur by a variety of mechanisms including nitro reduction, ring oxidation or a combination of these (1).

Similarly, the mutagenicity and carcinogenicity of arylamines has been studied extensively and convincing evidence has been presented that the primary pathway is oxidation to the corresponding arylhydroxylamines which are either direct-acting or become so following esterification to strong electrophiles (7,8,9). Thus arylhydroxylamines, or their hydroxamic acid esters, are considered the common mutagenic (and carcinogenic) intermediates of nitro- and aminoarenes (7,8,10-12). Heretofore esterification had not been considered as being mediated by a specific enzyme, however, evidence suggesting that in bacteria this process may be controlled by a specific gene product is presented herein .

Materials and Methods

2-Nitrofluorene, 2-aminofluorene, N-AcAAF, N-hydroxy-2-AAF, 2-aminoanthracene, BMBA and DMBA were obtained from the National Cancer Institute Chemical Repository at IIT Research Institute, Chicago (IL), 2-nitrosofluorene from Dr. Snorri S. Thorgeirsson, National Cancer Institute, quercetin from Pfaltz and Bauer, niridazole from Ciba-Geigy, and ascorbic acid from Sigma Chemical Co. S9 was prepared (13) from the livers of Syrian hamsters (Jackson Laboratories).

N-Hydroxy-2-aminofluorene was generated in situ from 2-nitrosofluorene by the addition of ascorbic acid (final concentration 5mM) (14).

Salmonella typhimurium strain TA98 was obtained from Dr. Bruce N. Ames, University of California (13). The isolation of TA98NR, a strain deficient in the "classical" nitroreductase was described earlier (15-17). This strain lacks a nitroreductase required for the activation of nitrofurans, nitroimidazoles, nitrofluorenes and other nitroarenes to mutagens (6,16,17). Strain TA98/1,8-DNP$_6$ was isolated as resistant to the mutagenicity of 1,8-dinitropyrene (18). This strain is also resistant to the mutagenicity of nitrofluorenes and other nitroarenes. It is entirely susceptible to the mutagenicity of nitrofurans and nitroimidazoles. These newly derived strains retain plasmid pKM101, the deep rough character (rfa) and their susceptibility to mutagenesis by non-nitrated polycyclic aromatic hydrocarbons and quercetin (see Table 1).

Mutagenicity assays were carried out as described by Ames and his associates (13). Every chemical was tested on at least two separate occasions and every dose level was assayed in triplicate. Mutagenic activity was calculated from the linear (ascending) portion of the dose response curve.

Table 1

Mutagenicity of 2-Hydroxylaminofluorene and Derivatives

Chemical	Revertants per Nanomole		
	TA98	TA98NR	TA98/1,8DNP$_6$
2-Nitrofluorene	84	2	2
2-Nitrosofluorene	542	504	79
N-Hydroxy-2-aminofluorene*	1175	1266	31
2-Aminofluorene + S9	154	127	15
N-Hydroxy-2AAF	371	343	20
N-AcAF	10	9	9
2-Aminoanthracene	494	546	25
DMBA	2	2	2
BMBA	14	15	17
Quercetin	0.9	1.1	1.0
Niridazole	400	17	400

*Obtained by the in situ reduction of 2-nitrosofluorene by ascorbic acid.

Results and Discussion

In accordance with previously published findings (1,19,20), both 2-nitrofluorene and 2-nitrosofluorene were mutagenic for Salmonella typhimurium TA98 (Table 1). The specific activity of 2-nitrosofluorene was higher than that of 2-nitrofluorene, as would be expected of a penultimate or ultimate mutagen.

While, in accordance with previously published results, the mutagenicity of 2-nitrofluorene was diminished in TA98NR, the strain deficient in "classical" nitroreductase, the activity of 2-nitrosofluorene was almost undiminished in that strain (Table 1). This finding, which confirms the results of Wirth et al. (20), suggests either that 2-nitrosofluorene is a direct-acting mutagen or that it requires further metabolism by a bacterial enzyme other than the "classical" nitroreductase. It is, therefore, significant that the mutagenicity of 2-nitrosofluorene is greatly reduced in strain TA98/1,8-DNP$_6$, a strain isolated as resistant to 1,8-dinitropyrene and which is also cross-resistant to other nitroarenes but displays susceptibility to many other chemical species. 2-Nitrofluorene, on the other hand, as previously reported (18), exhibited reduced mutagenicity in TA98/1,8-DNP$_6$.

These results suggest the possibility that the enzyme absent in TA98/1,8-DNP$_6$ is a nitrosoreductase and that the reduction of nitro-containing chemicals to mutagenic hydroxylamines may involve two separate enzymes. If indeed the enzyme deficiency in TA98/1,8-DNP$_6$ involves a nitrosoreductase, then it can be predicted that the block would be circumvented by N-hydroxy-2-aminofluorene. This was, however, not the case

as evidenced by the fact that in situ chemically-generated N-hydroxy-2-aminofluorene [ascorbic acid reduction of nitrosofluorene (14)] (Table 1) or zinc and ammonium chloride reduction of 2-nitrofluorene (21) (results not shown) did not by-pass the lesion present in TA98/1,8-DNP$_6$. As predicted from the bioactivaton pathway, N-hydroxy-2-aminofluorene exhibited an enhanced mutagenicity for strains TA98 and TA98NR. This further confirms that the lesion in TA98NR involves a nitroreductase-deficiency.

The greatly decreased mutagenicity of N-hydroxy-2-aminofluorene for TA98/1,8-DNP$_6$ was confirmed further by generating this chemical enzymatically from N-hydroxy-2-AAF, taking advantage of the deacetylase (or acyltransferase activity present in hamster S9) (20,22; see also 23,24). Additional evidence in support of this lack of mutagenicity was obtained from the S9-mediated oxidation of 2-aminofluorene (25) to N-hydroxy-2-aminofluorene (Table 1). While all of these procedures demonstrated the mutagenicity of N-hydroxy-2-aminofluorene for TA98NR, none of them caused the lesion in TA98/1,8-DNP$_6$ to be by-passed. On the other hand, the esterified electrophilic species N-AcAAF was equally active in all the strains (Table 1).

{Although N-AcAAF is equally active in all the strains (Table 1), its lower mutagenic activity when compared to the nitro- and hydroxylamino- and S9-activated derivatives is to be noted. This decreased activity of N-AcAAF has been observed on a number of previous occasions (16,26-29) and is most unexpected of an "ultimate" mutagen when compared to its precursors. It is, however, plausible that this difference is due to the fact that while all of the other mutagens tested are known to yield the 2-aminofluorene derivative of guanine (N-(deoxyguanosin-8-yl)-2-aminofluorene), N-AcAAF yields the N-acetyl derivative N-(deoxyguanosin-8-yl)-2-acetylaminofluorene. These two adducts induce very different conformational changes: the anti configuration for the unacetylated adduct and the syn configuration accompanied by a shift to the Z-DNA conformation for the acetylated adduct. These structural dissimilarities result in different rates of DNA repair and suggests that the Z-DNA conformation may ultimately lead to a decreased genetic and carcinogenic potency (30-36).}

These findings suggest that the lesion in strain TA98/1,8-DNP$_6$ is between the arylhydroxylamine and the ultimate mutagen. Because the ultimate mutagenic species

has been postulated as being an electrophilic ester and since, in fact, the ester N-AcAAF is fully active in TA98/1,8-DNP6, the present results suggest that the esterification of arylhydroxylamines in Salmonella does not occur spontaneously or through non-specific enzymes but that it is mediated by a specific enzyme, this putative enzyme being defective or absent in TA98/1,8-DNP6. Because the mutagenicity of non-nitrated polycyclic aromatic hydrocarbons (e.g., DMBA and BMBA) and of niridazole and quercetin is fully expressed in TA98/1,8-DNP6, while that of S9-activated 2-aminoanthracene (Table 1) is depressed, it is probable that this putative enzyme esterifies a variety of arylhydroxylamines. Both the specificity and distribution of this enzyme are under investigation.

As a number of nitroarenes display considerable activity in TA98/1,8-DNP6 while being blocked in TA98NR (e.g., 1-nitropyrene) (37,38), it may be hypothesized further that either some nitroso- or hydroxylamino- intermediates are direct-acting in their own right or that they become so following spontaneous dismutation to the nitrenium ion and that esterification is not required for their mutagenicity (8,39; see also 24,25,40,41). Finally, although the lesion in TA98/1,8-DNP6 has been identified as presumably involving an enzyme which esterifies arylhydroxylamines, the mutagenic specificities of other nitroarenes for TA98, TA98NR and TA98/1,8-DNP6 indicates the presence in Salmonella of additional enzymes, apparently other nitroreductases. Current studies of the properties of tester strains obtained by transposon-mediated mutagenesis, specifically resistant or hypersensitive to a variety of nitroarenes, may help identify these gene products.

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